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Growth-related changes in specific mRNAs of cultured mouse cells

(cDNA library/BALB/c 3T3 cells/cell growth/simian virus 40/platelet-derived growth factor)

DANIEL I. H. LINZER AND DANIEL NATHANS

Howard Hughes Medical Institute Laboratory, Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Contributed by Daniel Nathans, April 13, 1983

ABSTRACT **A cDNA plasmid library has been constructed from the poly(A)$^+$ RNA present in BALB/c 3T3 cells after serum stimulation. Of 3,500 clones tested, approximately 0.5% contained inserts corresponding to mRNAs present at higher levels in serum-stimulated BALB/c 3T3 cell cultures than in quiescent cultures. Most of these RNA species increased 2- to 5-fold, and the kinetics of increase for various RNAs differed. One clone (28H6) hybridized to a 1-kilobase RNA species that is present at barely detectable levels in resting cells but is increased at least 15- to 20-fold after serum stimulation, reaching a maximal level coincident with the onset of DNA synthesis. This RNA was at a high level in proliferating cells but decreased rapidly as cells reached confluence. 28H6 RNA was also increased in resting cells infected with simian virus 40 or stimulated with platelet-derived growth factor.**

Progression of cultured mammalian cells through the cell cycle appears to be regulated primarily during the G1 period—i.e., between the end of mitosis and the start of DNA replication (1). With cell lines such as the fibroblastic mouse BALB/c 3T3 line growing as a monolayer, growth ceases at confluence in G1 (2, 3) and can be reinitiated by serum-containing media or by purified growth factors (4, 5). Under these conditions stimulation results in a number of biochemical changes prior to the onset of a new round of DNA replication (6). Among these changes are increased uptake of certain ions and nutrients (7), incorporation of preexisting mRNAs into polyribosomes (8, 9), enhanced rRNA and tRNA (9–11) and protein (5, 6) synthesis, and increased transcription (5, 6) and processing (12) of mRNA precursors. Kinetic analysis of mRNA·cDNA hybridization has indicated that about 3% of the mRNA species found in growing mouse fibroblasts are absent from nongrowing cells (13). In addition to these overall changes in protein and RNA synthesis, there are characteristic changes in the levels or activities of specific proteins shortly after stimulation of resting cells. For example, enzymes involved in polyamine biosynthesis and DNA replication show an increase in activity (1), and specific proteins of unknown function are detectable by gel electrophoresis at characteristic times (e.g., see refs. 14 and 15). In view of the possible importance of specific gene activation during the transition from the G1 to S phase of growth, we have begun a study of individual mRNAs that are more abundant after serum stimulation of confluent BALB/c 3T3 cells. In this initial report we describe the preparation of a cDNA library from RNA isolated from stimulated cells at the onset of DNA replication and its use in the detection of mRNA species present at higher levels in growing than in resting cells.

MATERIALS AND METHODS

Enzymes and Growth Factors. All enzymes were purchased from commercial sources, except for avian myeloblastosis virus reverse transcriptase and *Escherichia coli* DNA polymerase I, which were kindly provided by J. Beard and P. Englund, respectively. Purified and titered platelet-derived growth factor (PDGF) was the generous gift of E. Raines and R. Ross (16).

Cell Culture. BALB/c 3T3 cells (17) were grown in Eagle's minimal essential medium with Earle's salts (GIBCO), supplemented with penicillin (10 units/ml), streptomycin (10 units/ml), glutamine (2 mM), and fetal bovine serum to 10% (MEM-10). Resting cultures were obtained by growing cells to confluence, feeding with minimal essential medium containing 0.5% (MEM-0.5) or 2% (MEM-2) fetal bovine serum, and maintaining the cells in low serum for at least 2 days. Resting cells were stimulated by feeding with minimal essential medium containing 20% fetal bovine serum (MEM-20), by adding PDGF directly to the medium at a final concentration of 11 ng/ml, or by infecting with CsCl-banded simian virus 40 (SV40) in MEM-0.5 at a multiplicity of 50 plaque-forming units per cell.

RNA Purification. RNA was prepared from guanidinium thiocyanate lysates of whole cells (18, 19), or from the cytoplasmic fraction of cells lysed in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/2.5 mM $MgCl_2$/0.5% Nonidet P-40 and digested with proteinase K at 100 μg/ml in the presence of 0.5% $NaDodSO_4$. RNAs were extracted with phenol/chloroform and precipitated with ethanol. Poly(A)$^+$ RNA was selected by two cycles of binding to oligo(dT)-cellulose (20).

Construction of the cDNA Library. Double-stranded cDNA was synthesized from approximately 20 μg of poly(A)$^+$ RNA (21) and inserted by G and C homopolymer tails (22, 23) into the unique *Pst* I site of the plasmid pKP43 [a 967-base-pair (bp) deletion mutant of pBR322 constructed and provided by K. Peden]. Annealed vector-cDNA was used to transform competent *E. coli* MM294 cells (24) to tetracycline resistance (25).

Colony Hybridization. Individual colonies were grown in L broth containing tetracycline at 4 μg/ml in 96-well microtiter trays and transferred to filters (GeneScreen, New England Nuclear) with a replica tool. Colonies were grown on the filters and the plasmid DNAs were amplified on L agar plates containing chloramphenicol at 250 μg/ml (26). Cells were lysed with 0.5 M NaOH, and the filters were washed with 1.0 M Tris·HCl, pH 7.4, and with 0.5 M Tris·HCl, pH 7.4/1.5 M NaCl (27). After baking and incubating as described (28), the filters were hybridized with cDNA probes at 1×10^6 dpm/ml for 48–72 hr at 68°C. ^{32}P-Labeled cDNA probes were synthesized to approximately 5×10^9 dpm/μg from cytoplasmic poly(A)$^+$ RNA from confluent cells maintained in MEM-0.5 for 6 days or from subconfluent cultures that were proliferating in MEM-10. Filters were washed (28) and autoradiographed (29).

Dot Blot Hybridization. Plasmid DNAs, linearized with

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Abbreviations: bp, base pair(s); kb, kilobase(s); MEM-x, minimal essential medium containing x% fetal bovine serum; PDGF, platelet-derived growth factor; pi, post infection; SV40, simian virus 40; T antigen, tumor antigen.

*Bam*HI restriction endonuclease, were denatured by heating in 0.1 M NaOH for 15 min at 100°C. Each sample was neutralized and immediately spotted on a nitrocellulose filter using a blotting manifold (Bethesda Research Laboratories). These dot blots were processed as described for the colony screen. The extent of hybridization was first analyzed by autoradiography and then quantified by liquid scintillation counting.

Preparation of Plasmid DNA. Recombinant plasmid DNAs were prepared on a small scale (30), or were purified by CsCl/ethidium bromide centrifugation (28). The chicken α-tubulin cDNA clone (31) was the generous gift of D. Cleveland.

RNA Filter Hybridization. RNA was electrophoresed on denaturing formaldehyde agarose gels (32, 33) and transferred to nitrocellulose (34). Filters were baked for 2 hr at 80°C under reduced pressure, incubated for 3–6 hr at 42°C in formamide buffer (35), and hybridized in fresh buffer supplemented with sonicated salmon sperm DNA at 10 μg/ml, tRNA at 5 μg/ml, and 1×10^6 dpm/ml of recombinant plasmid DNA nick-translated to $1–2 \times 10^8$ dpm/μg (36). After hybridization for 48 hr at 42°C, filters were washed (34), dried, and autoradiographed.

RESULTS

Construction of a cDNA Library from Serum-Stimulated Cells. Total cellular RNA was prepared from BALB/c 3T3 tissue culture cells at 12 hr after stimulation with MEM-20. This time corresponded to the onset of DNA synthesis as indicated by the incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material. (The maximal rate of DNA synthesis occurred 16–18 hr after the addition of serum.) The poly(A)$^+$ RNA fraction was used as template for the synthesis of double-stranded cDNA, which was inserted into the β-lactamase gene of the plasmid pKP43. The recombinant molecules were introduced into competent *E. coli*, generating a cDNA library of approximately 1×10^6 transformants.

Screening for Growth-Related Clones. Individual ampicillin-sensitive colonies were grown overnight in liquid culture in 96-well microtiter trays. Replica filters were prepared, colonies were established on the filters, and the plasmid DNA sequences were amplified by incubation of the filters in the presence of chloramphenicol. Plasmid DNA within each bacterial colony was denatured, immobilized on the filter, and hybridized to cDNA probes representing either resting or growing BALB/c 3T3 cell cytoplasmic poly(A)$^+$ RNA. The degree of hybridization was determined by autoradiography, as illustrated in Fig. 1*A*. Colonies that hybridized preferentially to the probe made from growing cell RNA were selected for further analysis. This initial survey eliminated approximately 95% of the 3,500 colonies screened; no colonies were identified that revealed consistently greater hybridization to the resting cell probe.

DNA was prepared from each clone harboring presumptive growing cell-specific sequences, as well as from a few control clones that gave no differential colony hybridization. Individual recombinant plasmid DNAs were prepared, applied in duplicate to nitrocellulose filters, and hybridized to resting and growing cell-specific probes. Autoradiography of these filters (Fig. 1*B*) revealed 13 clones that demonstrated preferential hybridization to the probe synthesized from growing cell RNA; these clones represent approximately 0.5% of the members of the library that were initially screened. The degree of differential hybridization of each of the 13 clones was quantified with CsCl-purified DNA dot blots. Denatured DNAs were spotted onto nitrocellulose, and the filters were hybridized to resting and growing cell-specific cDNA probes. The degree of hybridization was determined by measuring the cpm present in each dot in a liquid scintillation counter and subtracting the cpm bound to the corresponding amount of vector alone. The data

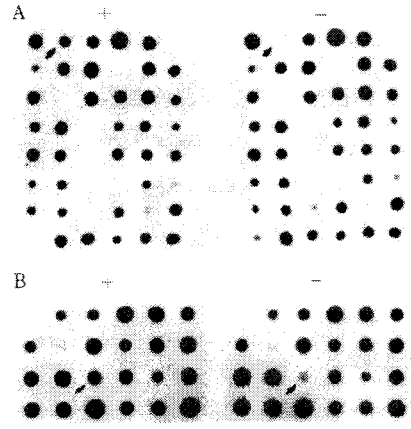

FIG. 1. Screening of the cDNA library. (*A*) Individual bacterial clones were grown in microtiter wells and transferred to duplicate filters, which were incubated on L agar plates supplemented with tetracycline at 4 μg/ml and then on L agar plates containing chloramphenicol at 250 μg/ml. Recombinant plasmid DNAs from lysed colonies were hybridized to cDNA probes synthesized from the cytoplasmic poly(A)$^+$ RNA present in actively growing BALB/c 3T3 cells (+) or in resting cells (−). The arrows indicate a particular clone (18A2) that demonstrated differential hybridization. (*B*) Recombinant plasmid DNAs were prepared from liquid cultures of individual bacterial clones (30), digested with *Bam*HI endonuclease, denatured with alkali, neutralized, and applied to nitrocellulose. Duplicate filters were hybridized to cDNA probes synthesized from the cytoplasmic poly(A)$^+$ RNA contained in growing (+) or resting (−) cells. The arrows indicate a clone (28H6) that hybridized preferentially to the growing cell RNA probe.

from these experiments as well as the size of each cDNA insert are summarized in Table 1. It is evident that the cloned cDNAs were derived from mRNAs that varied in response to serum and that differ in abundance.

Serum Stimulation of RNA Production. The eight individual clones that showed the greatest relative hybridization were uti-

Table 1. Summary of growing cell-specific cDNA clones

Clone	Insert, bp*	Hybridization, cpm†		Relative hybridization
		Growing cells	Resting cells	
32A4	250	490	52	9.4
18A2	225	685	75	9.1
28H6	450	1,650	230	7.2
20C2	900	552	94	5.9
16E11	250	820	159	5.2
17D2	225	1,314	270	4.9
20C8	200	384	79	4.9
18H3	725	2,166	640	3.4
24D10	325	676	301	2.2
24H4	150	677	355	1.9
34F10	250	490	267	1.8
24E7	375	524	323	1.6
23A4	425	83	65	1.3
16E5‡	200	650	689	0.9
26F5‡	225	424	444	1.0

* Insert sizes were measured on 2% agarose gels by comparing the mobilities of DNA fragments generated by at least two different restriction endonucleases to a set of standard fragments.

† Hybridization to growing cell-specific and resting cell-specific probes. Values presented are the average cpm of duplicate dots containing 250 ng of CsCl-purified plasmid DNA (or 450 ng in the case of 20C8); cpm contributed by hybridization to vector alone have been subtracted.

‡ Clones 16E5 and 26F5 demonstrated equal colony hybridization to the two probes, and they are presented here as controls.

lized to probe for levels of the corresponding RNA species in quiescent cells and at various times after serum stimulation. Total cellular RNA was prepared from confluent cells that had been maintained in MEM-2 and from cultures that were stimulated by feeding with MEM-20 for 6–36 hr. The RNAs were electrophoresed on formaldehyde/agarose gels, transferred to nitrocellulose, and probed with nick-translated cloned DNAs. Clones 18A2, 28H6, and 32A4 detected significant differences in the amount of the corresponding RNA present in stimulated versus nongrowing BALB/c 3T3 cell cultures (Fig. 2): 18A2 hybridized to a single size RNA of approximately 0.7 kilobases (kb); 28H6 detected a major RNA species of 1 kb and minor amounts of higher molecular weight RNA; and 32A4 hybridized to several RNAs. Fig. 2*D* represents a control hybridization employing a clone (31G8) that gave no differential hybridization in the colony and dot blot analyses. On the basis of intensity of hybridization to the various RNA species, it is likely that 28H6, 32A4, and 31G8 RNAs are more abundant than 18A2 RNA.

Each lane in Fig. 2 was traced with a densitometer, and the quantity of RNA detected at any given time was normalized to the maximal level attained for that RNA. As seen in Fig. 3, the time course of RNA levels differed for the RNAs examined. The level of 18A2 RNA remained low for 12 hr after serum stimulation, then rose sharply to at least 3 times the resting cell level and remained high through the 36-hr time point. (The sharp rise corresponded temporally to the onset of DNA synthesis.) By comparison, the 1-kb RNA that hybridized to the 28H6 probe increased steadily in amount to a peak at 12 hr after application of serum, reaching a level at least 15- to 20-fold higher than in resting cells. In other experiments, the 28H6 1-kb RNA was undetectable in resting cells and appeared within 3 hr after serum

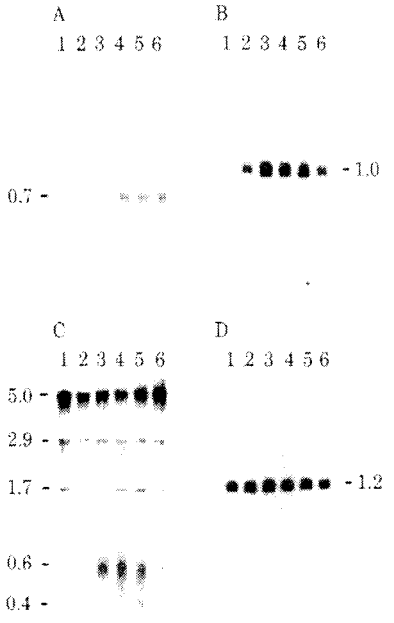

FIG. 2. Time course of the levels of specific RNA species after serum stimulation. Total cellular RNA (12 μg per lane) from resting and serum-stimulated cells was electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized to nick-translated probes. (*A*) The 18A2 probe, (*B*) 28H6, (*C*) 32A4, (*D*) 31G8. Lanes: 1, resting cells; 2, 6 hr after stimulation with MEM-20; 3, 12 hr; 4, 18 hr; 5, 24 hr; 6, 36 hr. The approximate sizes of the RNAs in kb were determined by comparison to 18S and 28S rRNA; note that the samples in *C* were electrophoresed for a longer time in order to resolve the multiple bands.

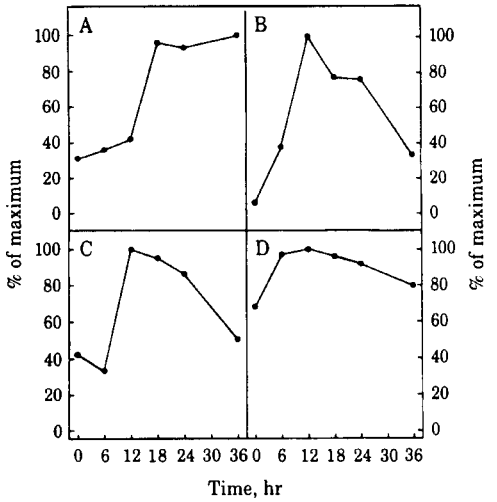

FIG. 3. Quantitation of RNA levels after serum stimulation. The optical density of each band in Fig. 2 was measured with an Optronics P1700 scanning densitometer, normalized to the maximal value obtained in each hybridization, and plotted versus the corresponding duration of serum stimulation. (*A*) The 0.7-kb 18A2 RNA, (*B*) 1-kb 28H6 RNA, (*C*) 0.6-kb 32A4 RNA, (*D*) 1.2-kb 31G8 RNA.

stimulation. Thus, the increase in 28H6-specific RNA preceded cellular DNA synthesis. After the 12-hr time point, 28H6 RNA decreased in quantity, again in contrast to the 18A2 RNA. The 32A4 probe revealed a more complicated pattern. As illustrated in Fig. 3*C*, the 0.6-kb RNA increased approximately 3-fold to a peak level at 12 hr and then decreased. The three larger RNA species remained constant in amount, while the 0.4-kb RNA level rose to a peak at 24 hr (Fig. 2*C*). Patterns of RNA identical to the pattern detected by the 32A4 clone were observed by using three of the other growth-specific clones as probes.

The poly(A)$^+$ fractions of the total cellular RNAs analyzed in Figs. 2 and 3 were also probed with the 18A2, 28H6, and 32A4 clones. The results were similar to those found for total cellular RNA. However, the higher molecular weight 28H6 RNA and the 32A4 0.4-kb and 2.9-kb bands were not detected in the poly(A)$^+$ fraction. Because of the virtual absence of 28H6 RNA from resting cells and the marked increase in the level of this RNA just before the onset of DNA synthesis, we decided to concentrate on 28H6 RNA in the experiments described below.

Growth State and RNA Levels. While the level of 28H6 1-kb RNA changed markedly upon serum stimulation of a confluent monolayer, it remained to be demonstrated that actively growing, subconfluent cultures have a higher concentration of this RNA than do resting cell cultures. Total cellular RNA was purified from BALB/c 3T3 cells maintained in MEM-10 and harvested at two subconfluent densities (approximately 20% and 90% confluent), during serum deprivation (1 and 2 days in MEM-0.5 after attaining confluence) or at 12 and 24 hr after feeding the 2-day-starved cultures with MEM-20. These RNAs were electrophoresed, transferred to nitrocellulose, and hybridized to a mixed probe of 28H6 plasmid DNA and an α-tubulin cDNA clone. As seen in Fig. 4*A*, the 1-kb RNA species that hybridized to the 28H6 cDNA was present at a high level in growing, subconfluent cell cultures (lane 1). This level decreased at the higher cell density (lane 2), even though the cells were still in medium containing 10% serum and the cultures had not quite reached confluence. As demonstrated above, starved cultures (lanes 3 and 4) also had a low concentration of this RNA, whereas serum-stimulated cells (lanes 5 and 6) expressed greatly increased amounts. The serum-stimulated sam-

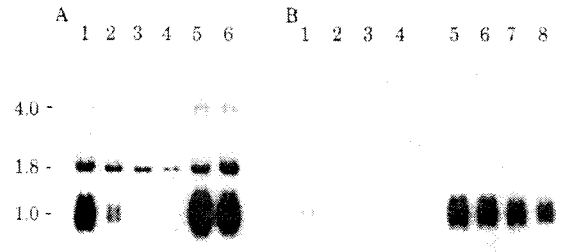

FIG. 4. Cell density and serum dependence of the level of 28H6 RNA. (*A*) Total cellular RNA (20 μg per lane), isolated from cell cultures in various states of growth, was electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with a mixed probe of nick-translated 28H6 DNA and cloned chicken α-tubulin cDNA. Lanes: 1, 20% confluent in MEM-10, 48 hr after plating; 2, 90% confluent in MEM-10, 96 hr after plating; 3, confluent and maintained for 24 hr in MEM-0.5; 4, confluent and maintained for 48 hr in MEM-0.5; 5, confluent, maintained for 48 hr in MEM-0.5, and fed with MEM-20 for 12 hr; 6, confluent, maintained for 48 hr in MEM-0.5, and fed with MEM-20 for 24 hr. (*B*) Cultures were fed with fresh MEM-2 or MEM-10 every 24 hr after plating in MEM-10 at a density of 3×10^3 cells per cm^2. Total cellular RNAs, prepared from cultures on days 2, 3, 4, and 5 after plating, were electrophoresed on a 1.5% agarose/formaldehyde gel (10 μg per lane), transferred to nitrocellulose, and hybridized to a nick-translated 28H6 DNA probe. Lanes: 1, MEM-2, day 2 (20% confluent); 2, MEM-2, day 3 (50% confluent); 3, MEM-2, day 4 (95% confluent); 4, MEM-2, day 5 (confluent); 5, MEM-10, day 2 (20% confluent); 6, MEM-10, day 3 (50% confluent); 7, MEM-10, day 4 (95% confluent); 8, MEM-10, day 5 (confluent).

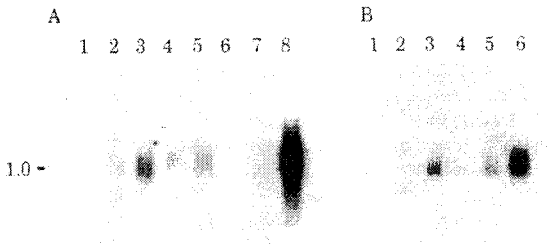

FIG. 5. Effect of purified mitogens on the level of 28H6 RNA. (*A*) Confluent cultures, maintained in MEM-0.5 for 6 days, were infected with CsCl-purified SV40 virions at 50 plaque-forming units per cell, mock-infected, or fed with MEM-20. Total cellular RNAs (25 μg per lane) were electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized to a nick-translated 28H6 DNA probe. Lanes: 1, uninfected; 2, 6 hr pi; 3, 12 hr pi; 4, 18 hr pi; 5, 24 hr pi; 6, 36 hr pi; 7, 24 hr after mock infection; 8, 12 hr after serum stimulation. (*B*) Confluent cultures of BALB/c 3T3 cells were starved in MEM-0.5 for 2 days, and PDGF in a solution containing bovine serum albumin (albumin) or albumin alone was added. Alternatively, cultures were fed with MEM-2, MEM-10, or MEM-20. Cells in all dishes were harvested for RNA isolation 12 hr after treatment. Total cellular RNA (20 μg per lane) was electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized to a nick-translated 28H6 DNA probe. Lanes: 1, untreated (i.e., resting in MEM-0.5); 2, albumin at 1.2 μg/ml; 3, albumin at 1.2 μg/ml and PDGF at 11 ng/ml; 4, MEM-2; 5, MEM-10; 6, MEM-20.

ples contained an increased quantity of the larger 28H6-specific RNA of 4 kb, as well. The α-tubulin 1.8-kb RNA also was present at a greater concentration in low-density, actively growing cell cultures than in higher-density and serum-depleted populations. Serum stimulation restored the amount of α-tubulin RNA to a quantity equal to or greater than that found in the 20% confluent cultures. It is clear, however, that the changes in the level of 28H6-specific RNA during this time course exceeded those observed for α-tubulin.

That a population of 90% confluent BALB/c 3T3 cells in MEM-10 contained low levels of 28H6 RNA suggested two possibilities: the RNA level might correlate directly with cell growth or cell density and be independent of serum concentration, or the RNA level might depend on an essential serum factor(s) that had been depleted during growth. To test these hypotheses, cells were plated at low density in MEM-10 and fed on the following day with either MEM-2 or MEM-10. On each successive day, some of the dishes were harvested for the preparation of total cellular RNA, while the medium in each of the remaining dishes was replaced with fresh MEM-2 or MEM-10, respectively. Cultures fed with MEM-2 and MEM-10 continued to grow and divide at the same rate during the course of the experiment. RNA samples were obtained from cultures that were about 20%, 50%, 95%, and 100% confluent. As shown in Fig. 4*B*, the amount of 28H6-specific RNA (both the 1-kb and 4-kb RNAs) was much greater in cells growing in 10% versus 2% serum, but the level decreased with increasing cell density in each case. Confluent cultures stimulated with fresh MEM-10 maintained a higher level of 28H6 RNA than did cultures that grew to near confluence in unchanged MEM-10 (compare Fig. 4 *A* and *B*). These results indicate that the level of 28H6-specific RNA varies both with the growth state or confluence of the cells and, more strikingly, with serum concentration.

Response to Defined Mitogens. The serum dependence of 28H6 RNA levels, as revealed in Fig. 4, suggested that the stimulatory factor(s) might be unrelated to the mitogenic activity of serum. Thus, the enhanced quantities of this RNA might not be a characteristic of BALB/c 3T3 cell growth but rather a response to nonmitogenic inducers. To determine whether defined mitogens affected the level of 28H6 RNA, we treated resting cultures with SV40 or PDGF, each of which has been shown to stimulate the growth of mouse 3T3 cells (37, 38).

Total cellular RNA was isolated from resting cultures of BALB/c 3T3 cells infected with purified SV40 virions at 6–36 hr post infection (pi), as well as from uninfected, 24-hr mock-infected, and 12-hr serum-stimulated cultures. After electrophoresis and transfer, the filter-bound RNA was probed with 28H6 DNA. As demonstrated in Fig. 5*A*, SV40 infection resulted in an increase of the level of 28H6 RNA, although to a lesser extent than serum stimulation (compare lanes 3 and 8). At least part of this difference can be accounted for by the relative resistance of starved 3T3 cells to infection by SV40. Even in infections with 50 plaque-forming units per cell, less than half of the cells expressed the SV40 large tumor (T) antigen, as assayed by indirect immunofluorescence. It is evident that the time course of the change in 1-kb RNA level in response to SV40 infection was similar to that observed for serum: the amount of RNA increased rapidly from an initial low level in uninfected (lane 1) or mock-infected (lane 7) cells to a peak level by 12 hr (lane 3), followed by a decrease at later times (lanes 4–6).

Cell cultures grown to confluence and maintained in medium with a low concentration of serum were also stimulated by addition of purified PDGF. At 11 ng/ml [5.5 ng/ml is equivalent to stimulating Swiss 3T3 DNA synthesis with 5% calf serum (E. Raines, personal communication)] PDGF markedly stimulated resting BALB/c 3T3 cells to synthesize DNA. In two independent experiments, this concentration of PDGF elicited a high level of 28H6 RNA (Fig. 5*B*). The PDGF-treated cultures produced more of this RNA than did cultures fed with MEM-10 (compare lanes 3 and 5), but less than cells stimulated with MEM-20.

DISCUSSION

To contribute to an understanding of the regulation of mammalian cell growth, we have initiated a study of specific mRNAs that increase in amount in serum-stimulated cells prior to or

coincident with the onset of cellular DNA synthesis. A similar approach has been taken recently to analyze the cellular effects of epidermal growth factor (39) and SV40 T antigen (40). Starting with a cDNA plasmid library derived from poly(A)$^+$ RNA isolated from stimulated BALB/c 3T3 cells at the time DNA replication begins, we have identified plasmids with cDNA inserts that hybridize better to a cDNA probe from growing cells than to a probe from resting cells. The original library consisted of 1×10^6 *E. coli* transformants and therefore should contain most of the cellular poly(A)$^+$ mRNA species that can be transcribed by reverse transcriptase. So far 3,500 clones have been screened. Of these, 13 clones detected mRNA species that were about 2- to over 15-fold enriched in growing cells; of the 13 positive clones, 4 appeared to recognize the same species of RNA. Therefore, distinct positive clones make up about 0.3% of the library. Plasmids with inserts representing the lowest-abundance class of RNAs probably would not have been detected by this screening procedure.

Specific RNAs identified with different cDNA-containing plasmids varied in their time of appearance and persistence in stimulated cells. Two RNAs reached their peak levels at about 12 hr after serum stimulation, at the time DNA synthesis commenced, and then decreased over the ensuing 24 hr. Another RNA species increased for about 18 hr and remained elevated. The marked and rapid increases seen for some species of RNA and the appearance of a new high molecular weight poly(A)$^-$ form in the case of 28H6 suggest that the increased levels are due, at least in part, to increased transcription.

Clone 28H6 was of particular interest because the corresponding 1-kb mRNA was under striking regulation. This RNA was barely detectable in resting cells, began to increase within 3 hr after serum stimulation, and reached high levels prior to the onset of DNA replication. 28H6 RNA also rapidly decreased as cells completed a serum-stimulated growth cycle or as a monolayer of growing cells reached confluence. However, growing cells transferred from high to low serum had much less of this mRNA than cells growing at a comparable rate in high serum concentration. Therefore, although the presence of this RNA correlates with the state of growth of BALB/c 3T3 cells, the amount present is also dependent on the concentration of serum.

Two other mitogens, SV40 and PDGF, also raised the level of 28H6 RNA. In the case of SV40, the virus-encoded T antigen is needed for stimulation of cell DNA synthesis (37), and we presume, but have not proven, that the rise in 28H6 RNA was also a T-antigen effect. Normalized for the number of T-antigen-positive cells, the level of 28H6 RNA in SV40-infected cells was similar to that seen after stimulation with 10% serum. In the case of PDGF, which is a major mitogen in serum (41), stimulation of 28H6 RNA was also comparable to that seen with 10% serum, suggesting that the serum effect was due in part to its content of PDGF. Although the presence of 28H6 RNA is correlated with cell growth induced by serum, PDGF, or SV40, it is not established that this RNA is an essential part of the growth process. However, the availability of cDNA clones for stimulated RNAs makes it possible to extend our investigation to the proteins encoded by these RNAs and to their structural genes, and eventually to introduce such genes into resting cells to determine their effect on cellular growth. Initial experiments along these lines indicate that 28H6 cDNA has an open reading frame coding for a protein with significant homology to mammalian prolactins. Whether this homology to a polypeptide hormone has any bearing on the role of 28H6 RNA in the cell growth cycle remains to be determined.

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